Skin-derived dendritic cells acquire and degrade the scrapie agent following *in vitro* exposure

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Summary

The accumulation of the scrapie agent in lymphoid tissues following inoculation via the skin is critical for efficient neuroinvasion, but how the agent is initially transported from the skin to the draining lymph node is not known. Langerhans cells (LCs) are specialized antigen-presenting cells that continually sample their microenvironment within the epidermis and transport captured antigens to draining lymph nodes. We considered LCs probable candidates to acquire and transport the scrapie agent after inoculation via the skin. XS106 cells are dendritic cells (DCs) isolated from mouse epidermis with characteristics of mature LC cells. To investigate the potential interaction of LCs with the scrapie agent XS106 cells were exposed to the scrapie agent *in vitro*. We show that XS106 cells rapidly acquire the scrapie agent following *in vitro* exposure. In addition, XS106 cells partially degrade the scrapie agent following extended cultivation. These data suggest that LCs might acquire and degrade the scrapie agent after inoculation via the skin, but data from additional experiments demonstrate that this ability could be lost in the presence of lipopolysaccharide or other immunostimulatory molecules. Our studies also imply that LCs would not undergo maturation following uptake of the scrapie agent in the skin, as the expression of surface antigens associated with LC maturation were unaltered following exposure. In conclusion, although LCs or DCs have the potential to acquire the scrapie agent within the epidermis our data suggest it is unlikely that they become activated and stimulated to transport the agent to the draining lymph node.

Keywords: dendritic cells; Langerhans cells; scrapie; skin; transmissible spongiform encephalopathy

Introduction

The transmissible spongiform encephalophathies (TSEs or prion diseases) are infectious, fatal, neurodegenerative diseases. During disease, PrP^{Sc}, an abnormal, relatively proteinase-resistant isoform of the host glycoprotein PrP^{C} accumulates with infectivity in affected tissues.[1] PrP^{Sc} is considered to constitute a major, or possibly sole component of the infectious TSE agent.[2] The deposition of PrP^{Sc} within the brains of most TSE-affected hosts is usually accompanied with neurodegeneration.

Many natural infections with TSE agents, such as natural sheep scrapie, bovine spongiform encephalopathy, chronic wasting disease in mule deer and elk and variant Creutzfeldt–Jakob disease (vCJD) in humans, are likely to be acquired orally. However, studies in mice show that skin scarification is also an effective means of scrapie transmission.[3] This suggests that some natural TSE cases might be transmitted through skin lesions in the mouth[4] or through sites of skin trauma during close contact with infected animals. The potential to transmit vCJD in humans iatrogenically during surgical or dental procedures is a current concern.

Following inoculation of mice with the scrapie agent via the skin,[5,6] peritoneal cavity[7,8] or orally[9] neuroinvasion usually occurs after the agent accumulates in association with follicular dendritic cells (FDCs) in the germinal centres of lymphoid tissues.

How TSE agents are transported initially from the site of exposure, such as the gastrointestinal tract or the skin, to the germinal centres where they replicate is not known. FDCs could directly trap PrP^{Sc} or other agent-associated molecules in a complement-bound complex.[10,11] TSE agents might also be delivered to germinal centres following

their uptake and transportation by mobile cells. Migratory bone marrow-derived DCs are a distinct lineage from tissue-fixed, stromal-derived FDCs.[12] These DCs circulate continually throughout the host's tissues and tissue fluids sampling antigens and transporting them to lymphoid tissues.[13] Langerhans cells (LCs) are a subset of migratory DCs that are situated within the epidermis and migrate to the draining lymph node following antigen encounter.[13] DCs and LCs are considered to provide potential mechanisms for the transmission of some viruses into the skin.[14,15] A subpopulation of migratory DCs might also play a role in the transportation of PrP^{Sc} from the intestine to the mesenteric lymph node.[16] These observations suggested to us that LCs were plausible candidates for the transportation of the scrapie agent from the skin to draining lymph nodes. XS106 cells are a well-established DC cell line isolated from mouse epidermis with characteristics of mature LCs. These cells strongly resemble LCs *in vivo* and potently activate naive T lymphocytes in allogeneic mixed lymphocyte reactions.[17] Our preliminary investigations suggested that XS106 cells had the potential to acquire and degrade PrP^{Sc}.[18] In the current study these cells were used to further explore the potential interactions of LCs with the scrapie agent following *in vitro* exposure.

Materials and methods

Culture of cell lines

The XS52 and XS106 cell lines (kind gifts from Professor Akira Takashima, University of Texas South-western, TX) are long-established dendritic cell lines derived from the epidermis of newborn mice.[17,19] These cell lines were cultured in complete RPMI-1640 medium (cRPMI) which contained 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 0·25 μg/ml antibiotic–antimycotic solution (A5955, containing penicillin, streptomycin and amphotericin B), 50 μM 2-β-mercaptoethanol, 1% non-essential amino acids (all from Sigma, Poole) and 10% heat-inactivated fetal calf serum (Invitrogen, Paisley). The medium used to cultivate the XS52 cells was supplemented further with 2 ng/ml murine recombinant granulocyte-macrophage colony stimulating factor (GM-CSF; R&D Systems, Abingdon, Oxon) and 10% (v/v) culture supernatant derived from the NS47 fibroblast cell line. The medium used to cultivate the XS106 cells was supplemented with 0·5 ng/ml murine recombinant GM-CSF and 5% (v/v) culture supernatant derived from the NS47 fibroblast cell line. All cell lines were cultured at 37° in a 5% CO_2/air atmosphere.

Upon receipt, cells were passaged four times to obtain a large batch culture and then cryopreserved. For each individual experiment a new vial of cells was revived and expanded for 10 days in culture prior to use to ensure homogeneity between cell populations.

Flow cytometry

Cells were harvested and viable cell numbers determined by trypan blue dye exclusion. Cells were pelleted (300 ***g*** for 10 min at 4°) and resuspended in FACS buffer [phosphate buffered saline (PBS) pH 7·4 containing 0·1% bovine serum albumin (BSA), 0·1% sodium azide and 0·02% ethylene diamine tetra-acetic acid (EDTA)] to give 1×10^6 cells per 100 μl buffer. Non-specific binding of immunoglobulins to FcRIII and FcRII receptors was blocked with 0·1 μg of mouse monoclonal antibody (mAb) CT-17·2 specific for CD16/32 (FcRIII/FcRII receptor; Caltag, Burlingame, CA). Cells were then incubated with one of the following fluorescein isothiocyanate (FITC)-conjugated surface marker-specific mAbs for 1 hr at 4°: CD40 (clone 3/23), CD80 (clone RMMP-1), CD86 (clone RMMP-2), major histocompatibility complex (MHC) II-A^k (clone 14 V18), CD205 (clone NLDC-145) (all from Serotec, Oxford), CD11c (clone HL3) or CD54 (clone 3E2) (BD Biosciences, Oxford). Immunolabelling for PrP was carried out using the PrP-specific mAb 8H4[20] followed by FITC-conjugated goat antimouse antibody (Caltag). Prior to their use in experiments all the above monoclonal antibodies were titrated to determine the concentration which would give 100% saturation. Appropriate FITC-conjugated antibodies were used as non-specific Ig-isotype controls (Serotec). All samples were fixed in 1% paraformaldehyde and flow cytometry conducted on a FACSCalibur™ (Becton Dickinson, San Jose, CA) and data analysed using CELLQuest™ software (Becton Dickinson).

mRNA isolation and cDNA synthesis

mRNA was isolated from XS106 cells using the μMACS mRNA Isolation Kit (Miltenyi Biotec Ltd, Bisley, Surrey) according to the manufacturer's instructions. To synthesize cDNA, 1 μl (25 μg) oligo $(dT)_{12-18}$ primer (Invitrogen) and 12 μl diethyl pyrocarbonate (DEPC)-treated water was added to 100 ng of the isolated mRNA and incubated at 70° for 10 min; 4 μl 5× first strand buffer, 2 μl dithiothreitol (DTT) (0·1 M) and 1 μl dNTP (10 mM; Invitrogen) were then added and incubated at 42° for 2 min. Finally 1 μl of Superscript™ II reverse transcriptase (Invitrogen) was added and incubated at 42° for 50 min. The reaction was stopped by incubation at 70° for 15 min.

Analysis of PrP mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

The PCR mixture (total volume 60·2 μl) contained; 5 μl 10× PCR buffer, 1·5 μl 50 mM MgCl, 1 μl 10 mM dNTP mix (Invitrogen), 1 μl cDNA, 0·5 μl (100 pmol/μl) of each specific primer, 0·2 μl *Taq* polymerase (Invitrogen) and 50 μl sterile DNase and RNase free water. The sequences of the specific primers used were as follows:

Primer combination 1

(1) PrP Fwd-5′-AGG TTA ACT GAA CCA TTT CAA CCG AGC-3′
(2) PrP Rev-5′-TCC CCC AGC CTA GAC CAC GAG A-3′

Primer combination 2

(1) PrP Fwd-5′-AGG TTA ACT GAA CCA TTT CAA CCG AGC-3′
(3) PrP Rev-5′-GCT TGT TCC ACT GAT TAT GG-3′

Following a hot start at 94° for 3 min an amplification cycle was carried out for 30 cycles at the following temperatures on a thermal cycler: 94° 30 seconds, 63° 30 seconds, 72° 60 seconds. A final extension period at 72° for 10 min was included at the end of the 30 cycles. PCR products were resolved by electrophoresis at 125 V through a 1·5% agarose gel containing 1 μg/ml ethidium bromide. Primers 1 and 2 are designed to generate a 1000 base pairs (bp) fragment; primers 1 and 3 generate a 400 bp fragment.

Analysis of Toll-like receptor (TLR)-2 and TLR-4 mRNA expression by RT-PCR

The PCR mixture (total volume 60·2 μl) contained: 5 μl 10× PCR buffer, 1·5 μl 50 mM MgCl, 1 μl 10 mM dNTP mix, 1 μl cDNA, 0·5 μl (100 pmol/μl) of each specific primer, 0·2 μl *Taq* polymerase and 50 μl sterile DNase and RNase free water. The sequences of the specific primers used were as follows:

TLR-2 Fwd-5′-GTCTCTGCGACCTAGAAGTGGA-3′
TLR-2 Rev-5′-CGGAGGGAATAGAGGTGAAAGA-3′
TLR-4 Fwd-5′-GCAATGTCTCTGGCAGGTGTA-3′
TLR-4 Rev-5′-CAAGGGATAAGAACGCTGAGA-3′

Following a hot start at 94° for 2·5 min an amplification cycle was carried out for 32 cycles at the following temperatures on a thermal cycler: TLR-2, 94° 30 seconds, 56° 30 seconds, 72° 45; TLR-4, 95° 45 seconds, 61° 45 seconds, 72° for 45 seconds, for 36 cycles. A final extension period at 72° for 10 min was included at the end of the cycles. PCR products were resolved by electrophoresis at 125 V through a 1·5% agarose gel containing 1 μg/ml ethidium bromide. Primers for TLR-2 are designed to generate a 336 bp fragment; primers for TLR-4 are designed to generate a 406 bp fragment.

Exposure of cells to scrapie

For scrapie inoculation studies, sterile glass coverslips were placed in the wells of a flat-bottomed 24-well plate (Corning, Buckinghamshire). Cells (5×10^5 in 1 ml of cRPMI) were seeded into each well. When confluent (approx. 2×10^6 cells/coverslip), each well was inoculated with 10 μl of 10% (wt/vol) brain homogenate from a terminally ME7 scrapie-affected C57BL/Dk mouse (equivalent to 1·0 mg tissue). Duplicate sets of culture cells were inoculated with 10 μl of 10% (wt/vol) uninfected mouse brain homogenate in cRPMI as a control. The medium was removed after 16 hr incubation at 37° in a 5% CO_2/air atmosphere and cells in each well washed extensively with fresh medium by pipetting. Cells were then maintained for the times indicated before further analysis. Where indicated some cells were stimulated with 10 ng/ml of lipopolysaccharide (LPS, serotype 055:B5; Sigma) 1 hr before exposure to the scrapie agent.

Immunofluorescent confocal microscopy

Following exposure to the scrapie agent, cells were washed and cytospin preparations prepared (approximately 1×10^5 per slide). Cytospins were fixed in methanol at −20° for 20 min and non-specific binding sites blocked with 3% BSA for 1 hr. Non-specific binding to Fc-receptors was blocked by addition 0·1 μg of mouse mAb CT-17·2 (Caltag). Avidin/biotin binding sites were blocked subsequently using the avidin/biotin blocking kit (Vector Laboratories, Peterborough) according to the manufacturer's instructions. PrP was detected using the PrP-specific mouse mAb 8H4[20] followed by biotin-conjugated goat antimouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunolabelling was carried out using Alexa 488-conjugated streptavidin (Biosciences, Cambridge). Cell nuclei were distinguished using DAPI nucleic acid stain (Biosciences).

Cell immunoblot detection of PrP^{Sc}

PrP^{Sc} accumulations were determined by the cell immunoblot procedure.[21] Briefly, coverslips were removed and the cell-bearing side blotted onto polyvinylidine difluoride membranes (Bio-Rad, Hemel Hempstead), treated with proteinase K, denatured with guanidine isothiocyanate and PrP detected using the PrP-specific mAb 8H4.[20] Immunolabelling was carried out using horseradish peroxidase-conjugated goat antimouse IgG antiserum (Jackson ImmunoResearch Laboratories Inc.), and bound horseradish peroxidase activity detected with Supersignal® West Dura Extended Duration Substrate (Pierce, Chester). After exposure, membranes were stained with 0·5 μg/ml ethidium bromide and observed in UV light to confirm transfer of the cell layer.[22]

Determination of scrapie agent infectivity

Following exposure to the scrapie agent for the times indicated cells were dislodged using a rubber policeman and washed twice in Hanks's balanced salt solution

(HBSS). Viable cell numbers were determined by trypan blue dye exclusion. Cell pellets were resuspended in an appropriate volume of sterile PBS to give 2×10^5 cells per 20 µl dose. Cells were lysed by 10 successive rapid freeze–thaw cycles at the temperature of liquid nitrogen. Non-infected cells or cells exposed to normal brain homogenate were used as reference controls. Cell lysates (20 µl) were injected intracranially (i.c.) into groups of nine C57BL/Dk indicator mice. The scrapie titre in each lysate was estimated from the mean incubation period from a dose/incubation period response curve for scrapie-infected spleen tissue.[23] Infectivity titres are expressed as 50% infectious dose units per 10^6 cells ($ID_{50}/10^6$ cells).

Statistical tests

Data from individual experiments are presented and are representative of data from two to five experiments. Where applicable, the statistical significance of differences between the means for each experimental group were calculated using ANOVA one-way analysis with Minitab software. A P-value = 0·05 was considered to be significantly different.

Results

Characterization of the XS106 cell line

Prior to use in experiments the surface phenotype of the XS106 cells was determined. FACS analysis confirmed that the XS106 cells expressed the following surface markers: CD11c, CD40, CD54, CD80, CD86, CD205 and Ia^k (MHC-II) (Fig. 1b–h, respectively). Daily analysis of XS106 cells for 4 further days in culture demonstrated that this surface phenotype remained unchanged during the observation period (data not shown).

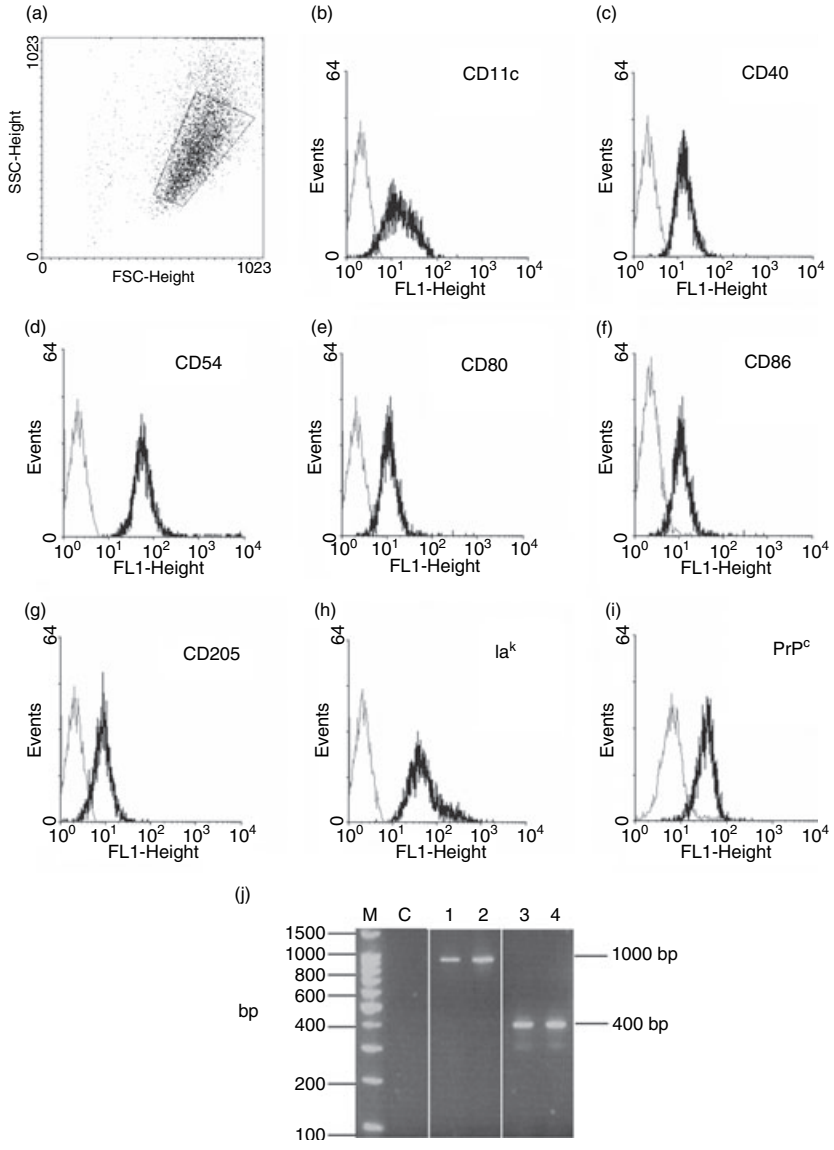

Figure 1. (a–i) FACS analysis of the surface antigen phenotype of XS106 cells. XS106 cells were gated as shown (a). Cells were stained with a panel of FITC-conjugated mAbs specific for the following antigens: CD11c (b), CD40 (c), CD54 (d), CD80 (e), CD86 (f), CD205 (g), Ia^k (h) and PrP (i). Bold histograms represent cells stained with surface antigen-specific mAbs, fine histograms represent cells stained with isotype-matched, non-specific FITC-conjugated control mAbs. (j) XS106 cells express mRNA for PrP^C. RT-PCR analysis confirmed the presence of PrP^C-specific mRNA by the visualisation of a single band at 1000 bp (lanes 1 and 2; using primer combination (1) and also a single band at 400 bp (lanes 3 and 4; using primer combination (2). Lane M, 100 bp molecular size markers. Lane C, non-reverse transcriptase treated mRNA preparation from XS106 cells as a control.

XS106 cells express PrP^C

Next, the expression of PrP^C protein and mRNA by XS106 cells was determined. FACs analysis demonstrated that the majority of the XS106 cells expressed PrP^C protein on their surfaces (Fig. 1i). The expression of PrP-specific mRNA was confirmed using two different sets of primers by the visualization of a single band at 1000 bp using primer combination 1 (Fig. 1j, lanes 1 and 2) and also a single band at 400 bp using primer combination 2 (Fig. 1j, lanes 3 and 4).

XS106 cells rapidly acquire scrapie-affected brain homogenate

We next determined whether XS106 cells could acquire the scrapie agent after *in vitro* exposure. Cells were exposed to terminally scrapie-affected brain homogenate for 10 min, 30 min, 16 hr or 24 hr. Uninfected cells and cells exposed to an equivalent amount of uninfected (normal) brain homogenate were included as controls. Immunofluorescent confocal analysis of cytospin preparations revealed abundant accumulations of PrP in association with the XS106 cells within 10 min of exposure to scrapie-affected brain homogenate (Fig. 2, panel c). Visually, the strongest labelling for PrP was detected after 30 min of exposure to the scrapie brain homogenate (Fig. 2, panel f). Thereafter, the intensity of PrP staining appeared to decrease during the 24-hr observation period (Fig. 2, panels i and l). Accumulations of PrP were also detected in association with XS106 cells exposed to normal brain homogenate over the same time period (Fig. 2, panels, b, e, h and k). In contrast, the level of PrP immunolabelling detected in association with unexposed cells (Fig. 2, panels a, d, g and j) was visibly less when compared to cells exposed to either scrapie brain homogenate or normal brain homogenates. Thus, these data appear to show that XS106 cells rapidly acquire scrapie-affected and normal brain homogenate after *in vitro* exposure. However, as the PrP-specific mAb 8H4 does not distinguish between the cellular PrP^C or scrapie agent-specific PrP^{Sc}

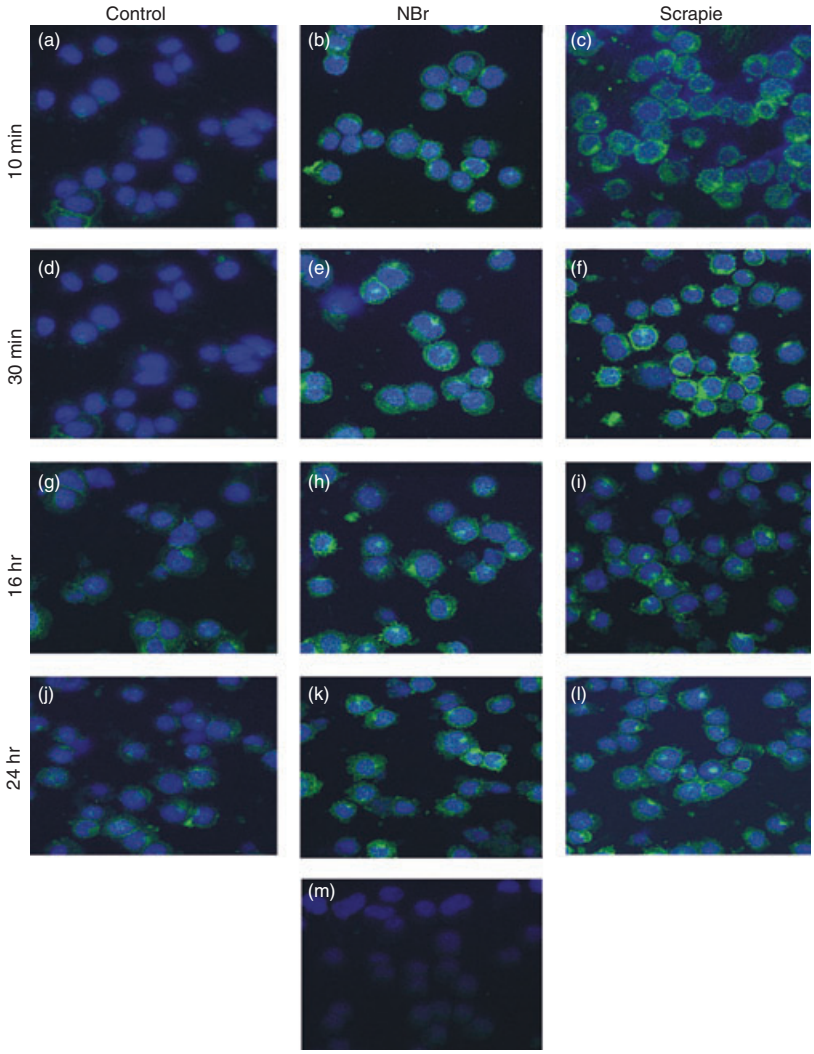

Figure 2. XS106 cells rapidly acquire scrapie brain homogenate. Immunofluorescent confocal analysis of cytospin preparations detected the presence of abundant intracellular accumulations of PrP (green) within XS106 cells exposed to scrapie brain homogenate (scrapie; panels c, f, i and l) when compared to unexposed cells (control; panels a, d, g and j). Intracellular PrP accumulations were also detected within XS106 cells exposed to normal brain homogenate (NBr; panels b, e, h and k). Cells were exposed to scrapie or NBr for 10 min (top row), 30 min (second row), 16 hr (third row) or 24 hr (bottom row) before analysis. (m) unexposed cells stained with an isotype-matched, non-specific FITC-conjugated mAb as a control. Original magnification ×40. All sections were counterstained with DAPI to detect cell nuclei (blue).

isoforms, it was not possible to determine immunocytochemically whether the PrP detected was PrP^{Sc}, PrP^{C} from the brain homogenate or enhanced expression of PrP^{C} by XS106 cells in response to the brain homogenate.

XS106 cells acquire and degrade PrP^{Sc} and infectivity

To investigate further whether XS106 cells acquire PrP^{Sc} following exposure to scrapie brain homogenate, cellular accumulations of agent-specific proteinase K (PK)-resistant PrP^{Sc} were analysed by cell immunoblotting. Monolayers of XS106 cells were cultured on sterile glass coverslips and then exposed to terminally scrapie-affected brain homogenate for 16 hr. Cells were then washed to remove excess brain homogenate and maintained in culture for up to 96 hr. Uninfected cells and cells exposed to normal brain homogenate were included as controls. Following inoculation, coverslips were removed and PrP^{Sc} levels determined by cell immunoblotting.

XS106 cells exposed to scrapie brain homogenate and assayed immediately after washing (16 hr) or 24 hr after exposure contained abundant levels of PK-resistant PrP^{Sc} (Fig. 3a). However, analysis of cells from 48 hr onwards suggested that the levels of PrP^{Sc} detected progressively decreased over the 96-hr observation period (Fig. 3a[18]). In contrast, no PrP^{Sc} was detected in association with cells exposed to normal brain homogenate (Fig. 3a) or unexposed cells (Fig. 3c) as controls. The successful transfer of cells to the membranes was confirmed by the detection of cellular DNA on membranes for each cell transfer (Fig. 3b). No PrP^{Sc} or DNA was detected in association with cell-free cover-slips exposed to scrapie brain homogenate as a control (Fig. 3d), confirming that the PrP^{Sc} detected in the above instances (Fig. 3a) was cell-associated and not simply adhered to the coverslip. No significant effect on cell viability or metabolic activity was observed following exposure of the cells to scrapie brain homogenate when compared to those exposed to normal brain homogenate or medium alone, as controls (data not shown). Thus the reduction in the PrP^{Sc} associated with the XS106 LC-like cells was unlikely to be due to reduced cell viability. A further experiment was included to determine whether the reduced detection of PrP^{Sc} in association with XS106 cells was due to masking the epitope recognized by mAb 8H4. The PrP-specific mAb 7A12 recognizes an epitope contained within amino acid residues 122–143 of the PrP protein which is distinct from the epitope recognized by mAb 8H4 (amino acids 147–164[20]). The level of PrP^{Sc} detected following exposure of XS106 cells to scrapie brain homogenate was similarly visibly reduced over the 96-hr observation period when mAb 7A12 was used to detect PrP^{Sc} (Fig. 3e).

To determine whether the degradation of PrP^{Sc} by XS106 cells correlated with a reduction in scrapie infectivity, lysates were prepared from XS106 cells exposed to scrapie-affected brain homogenate and infectivity titres determined by incubation period assay by i.c. injection into nine C57BL/Dk indicator mice. Lysates prepared from

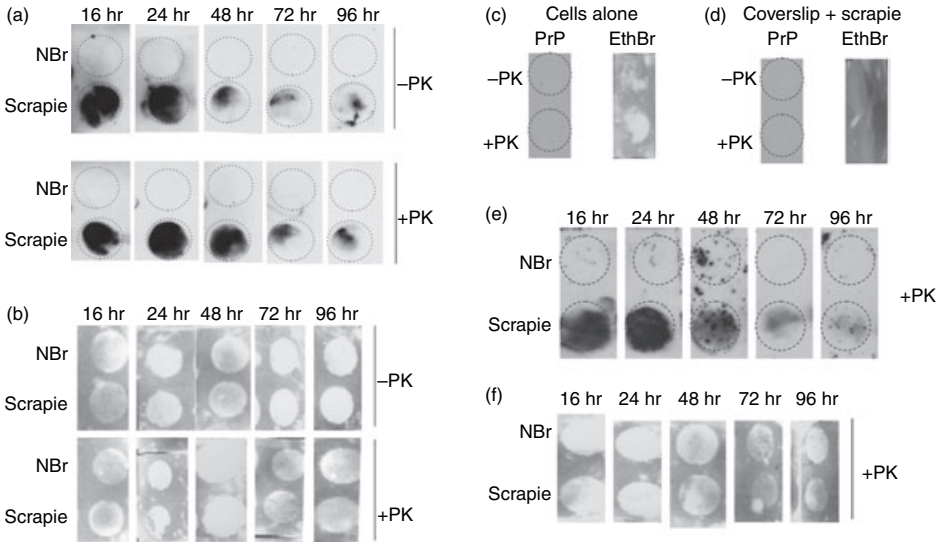

Figure 3. XS106 cells acquire and degrade PrP^{Sc}. Duplicate cultures of XS106 cells were exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (ME7) for 16 hr, washed and then maintained in culture for the times indicated. Cells were then transferred to PVDF membranes and treated in the absence (–) or presence (+) of PK before immunostaining with PrP-specific mAb 8H4 (a) or 7A12 (e). Cell immunoblot analysis showed a progressive decrease in the levels of PK resistant PrP^{Sc} associated with the scrapie-exposed XS106 cells over the 96-hr observation period (a and e). No PrP^{Sc} was detected in association with XS106 cells exposed to NBr (a and e), unexposed XS106 cells (c) or cell-free coverslips exposed to scrapie brain homogenate (d). Ethidium bromide (EthBr) staining of the PVDF membranes confirmed the transfer of cellular DNA (b, c and f). Panel (a) is adapted from Mohan *et al.*[18]

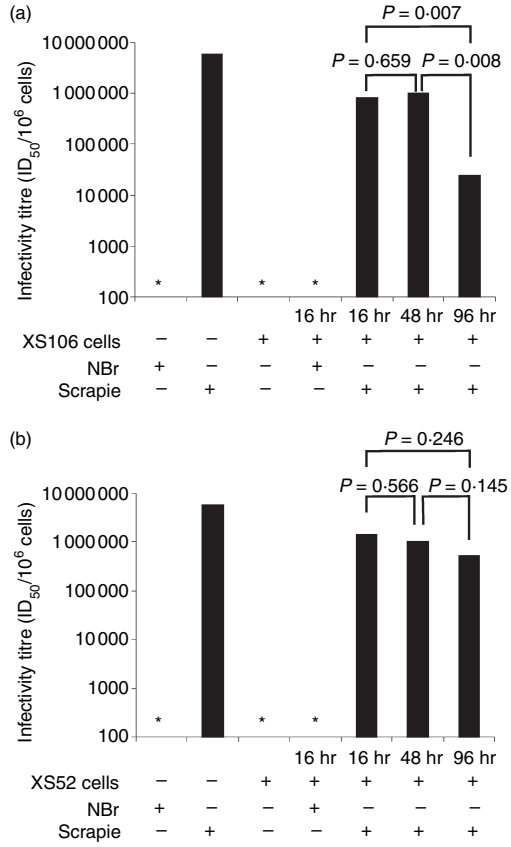

Figure 4. XS106 cells acquire and degrade scrapie agent infectivity. Duplicate cultures of XS106 cells (a) or XS52 cells (b) were exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (ME7) for 16 hr, washed and then maintained in culture for the times indicated. Cell lysates were then prepared and scrapie agent infectivity titres determined by incubation period assay by i.c. injection of the lysates into groups of nine indicator mice. Infectivity titres are expressed as $ID_{50}/10^6$ cells. *All indicator mice remained free of the signs of scrapie up to at least 400 days after inoculation suggesting an infectivity titre, if present, below 100 $ID_{50}/10^6$ cells.

uninfected XS106 cells and cells exposed to normal brain homogenate were included as negative controls. After 16 hr exposure to scrapie brain homogenate the cells had acquired approximately 5·9 log i.c. $ID_{50}/10^6$ cells (Fig. 4a). Statistical analysis of the means of the disease incubation periods for each group of indicator mice injected with homogenate prepared 16 hr (190 ± 2 days, $n = 9$) or 24 hr (188 ± 3 days, $n = 9$) after exposure suggested there was no significant difference between the infectivity titres associated with these cells at 16 hr and 24 hr postexposure ($P = 0{\cdot}659$, ANOVA, $n = 9$). However, by 96 hr after exposure the infectivity titre had declined to approximately 4·4 log i.c. $ID_{50}/10^6$ cells (Fig. 4a). Comparison of the mean disease incubation period obtained from mice injected with 96-hr homogenates (220 ± 9 days, $n = 9$) with those measured at both 16 hr and 24 hr postexposure suggested the infectivity titre measured after 96 hr exposure was significantly lower ($P = 0{\cdot}007$ and $P = 0{\cdot}008$, respectively, ANOVA, $n = 9$). Scrapie infectivity was undetectable in preparations of normal brain homogenate alone or lysates of uninfected cells or cells exposed to normal brain homogenate (Fig. 4a). These data confirm that the degradation of PrP^{Sc} by XS106 cells correlated with a significant reduction in the level of scrapie agent infectivity associated with these cells.

Effect of scrapie exposure on the surface expression of costimulatory and activation markers by XS106 cells

FACS analysis was used to determine whether the expression of specific surface antigens by XS106 cells was altered following exposure to the scrapie agent. The experiment demonstrated that exposure to the scrapie agent did not significantly change the expression of CD11c, CD40, CD54, CD80, CD86, CD205, Ia^k or PrP when compared to cells exposed to normal brain homogenate (Fig. 5). These data suggest that XS106 cells do not undergo further maturation or activation after *in vitro* exposure to the scrapie agent.

Immature LC-like cells do not degrade the scrapie agent *in vitro*

Unlike the XS106 cell line used above, the XS52 cell line is an immature LC-like cell line isolated from mouse epidermis.[19] FACS analysis confirmed that these cells do not express the surface antigens typically associated with LC maturation (CD40, CD80, CD86 and Ia^d; Fig. 6a–d, respectively), indicating that these cells displayed an immature phenotype when compared to XS106 cells (Fig. 1). However, XS52 cells expressed PrP^C protein (Fig. 6e). The XS52 cell line was used in parallel studies to determine whether the degradation of PrP^{Sc} by XS106 cells is typical of any *in vitro* cultivated cell line exposed to the scrapie agent. Under the same experimental conditions, XS52 cells accumulated abundant PrP^{Sc} after 16 hr exposure to scrapie brain homogenate (Fig. 6f). However, in contrast to XS106 cells exposed to the scrapie agent (Figs 3a and 4a), there was no progressive decrease in PrP^{Sc} (Fig. 6f) or infectivity (Fig. 4b) over the 96-hr observation period.

LPS inhibits the degradation of PrP^{Sc} by XS106 cells

As non-specific immune stimulation around the time of peripheral inoculation with TSE agents can enhance disease pathogenesis[24] we next investigated the effect of LPS stimulation on PrP^{Sc} degradation by XS106 cells. LCs can bind LPS via Toll-like receptor (TLR)-2 and TLR-4[25,26] in association with CD14.[27] RT-PCR analysis confirmed that XS106 cells expressed mRNA specific for both TLR-2 (Fig. 7a) and TLR-4 (Fig. 7b). The demonstration that XS

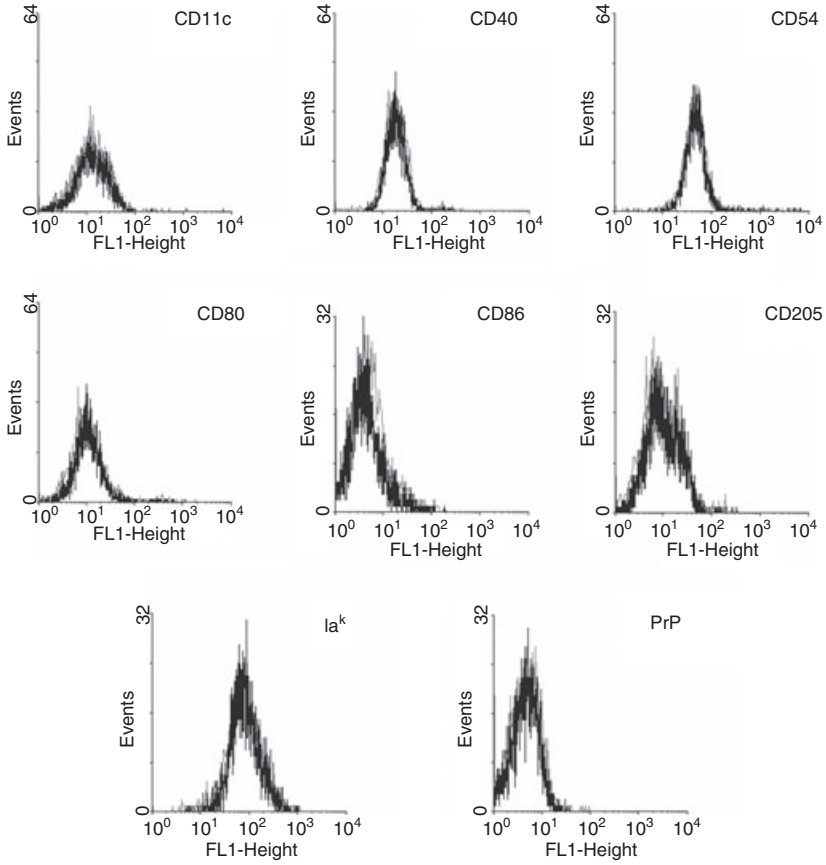

Figure 5. Effect of exposure to the scrapie agent on surface antigen expression by XS106 cells. Before FACS analysis cells were stained with a panel of FITC-conjugated mAbs specific for the following antigens: CD11c, CD40, CD54, CD80, CD86, CD205, Ia^k and PrP. Bold histograms represent cells exposed to scrapie brain homogenate, fine histograms represent cells exposed to normal brain homogenate.

cells also express CD14[28] indicates that these cells would have the potential to respond to LPS stimulation. LPS pretreatment appeared to block the degradation of PrP^{Sc} by XS106 cells, as cells stimulated with LPS prior to exposure to the scrapie agent showed no decrease in PrP^{Sc} over the 96-hr observation period (Fig. 7c).

Discussion

Here we show that LC-like XS106 cells rapidly acquire the scrapie agent following *in vitro* exposure. After uptake, the PrP^{Sc} and infectivity levels associated with the scrapie agent-exposed XS106 cells were significantly degraded with extended cultivation. These data suggest that LCs might acquire and degrade the scrapie agent after inoculation via the skin. Highly immature XS52 LC-like cells acquired but did not significantly degrade the scrapie agent following *in vitro* exposure. The ability of XS106 cells to degrade PrP^{Sc} was lost following stimulation with LPS implying that the ability of LCs to degrade PrP^{Sc} might be blocked in the presence of immunostimulatory molecules within the epidermis. Exposure to the scrapie agent did not alter the surface expression of PrP^{C} or a variety of costimulatory or activation markers on the XS106 cells. These data suggest that LCs or skin DCs have the potential to acquire and degrade the scrapie agent within the epidermis but would not undergo further maturation.

Following uptake by DCs, antigens are usually processed into short peptides for presentation to T lymphocytes in association with MHC. However, in some circumstances DCs can retain protein antigens in a native, non-degraded form.[29] The precise fate of the scrapie agent following uptake by DCs is unclear, as studies have shown these cells can retain[16] or degrade PrP^{Sc}.[30] Thus we investigated the actions of LC-like cells on the scrapie agent following *in vitro* exposure. Immunofluorescent confocal analysis demonstrated that, following exposure to scrapie brain homogenate, XS106 cells contained heavy intracellular deposits of PrP. Further experiments demonstrated that these PrP deposits contained PrP^{Sc} and infectivity which was subsequently degraded following extended cultivation. The mechanism through which XS106 cells degrade the scrapie agent is not known, but may be mediated by cysteine proteases as shown for the degradation of PrP^{Sc} by bone marrow-derived $CD11c^{+}$ myeloid DCs.[31] Accumulations of PrP^{Sc} and infectivity were also detected in association with the highly immature XS52 LC-like cell line following exposure to the scrapie agent. However, the levels of PrP^{Sc} and infectivity associated with this cell line were not subsequently degraded. These data imply that the ability of LCs or DCs to

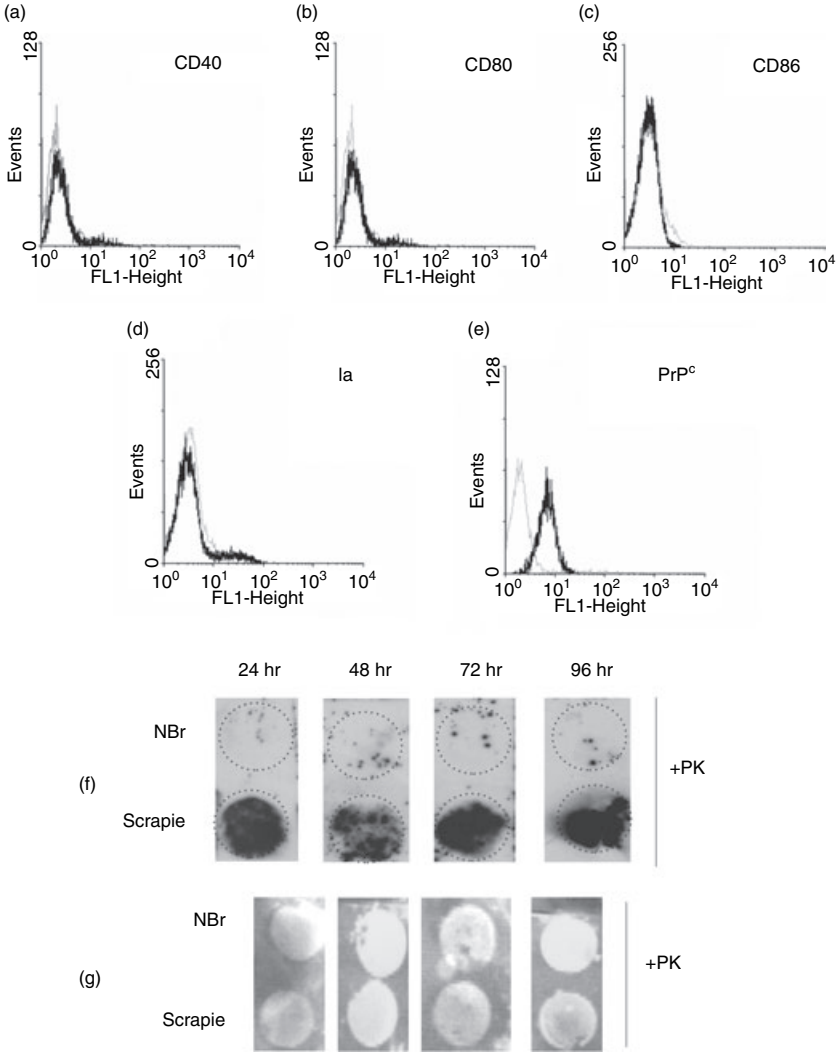

Figure 6. XS52 cells acquire but do not degrade the scrapie agent. (a–e) FACS analysis of the surface antigen phenotype of XS52 cells. Cells were stained with a panel of FITC-conjugated mAbs specific for the following antigens: CD40 (a), CD80 (b), CD86 (c), Ia^k (d) and PrP (e). Bold histograms represent cells stained with surface antigen-specific mAbs, fine histograms represent cells stained with isotype-matched, non-specific FITC-conjugated control mAbs. (f and g) Duplicate cultures of XS52 cells were exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (ME7) for 16 hr, washed and then maintained in culture for the times indicated. Cells were then transferred to PVDF membranes and treated in the absence (–) or presence (+) of PK before immunostaining with the PrP-specific mAb 8H4. Cell immunoblot analysis showed scrapie agent exposed XS52 cells acquired PrP^{Sc} but did not degrade it over the 96-hr observation period (f). Ethidium bromide staining of the PVDF membranes confirmed the transfer of cellular DNA (g). Panel (f) is adapted from Mohan *et al.*[18]

acquire and partially degrade the scrapie agent may be related to the maturation status of the cells.

Exogenous stimuli such as inflammatory cytokines,[32] CpG oligonucleotides[33] and LPS[34] induce the activation and maturation of LCs. Studies by Sethi *et al.*[35] have shown that treatment of mice with CpG oligonucleotides extended the survival time of scrapie agent-inoculated mice. The effect of CpG treatment on disease pathogenesis was considered to be due to the enhanced clearance of the scrapie agent by TLR-stimulated DCs or macrophages.[35] As LCs[26] and XS106 cells (Fig. 7) express TLR-2 and TLR-4 which bind LPS, we investigated whether LPS-stimulation would enhance the degradation of PrP^{Sc} by XS106 cells. However, we found that LPS-stimulation blocked the degradation of PrP^{Sc} by XS106 cells. These data appeared to be in opposition to the hypothesis that TLR-mediated stimulation of the innate immune system enhances the degradation of the scrapie agent.[35] However, subsequent data show that the CpG oligonucleotide treatment protocol used by Sethi *et al.*[35] destroys lymphoid follicles,[36] the main peripheral site of replication for the scrapie agent.[7,8] Furthermore, it is unlikely that TLR-mediated stimulation plays an important role in the degradation of the scrapie agent as disease pathogenesis is unaffected in *Myd88*$^{-/-}$ mice with defective TLR signalling.[37] The mechanism through which LPS blocks the degradation of PrP^{Sc} by XS106 cells is unknown. Because LPS-induced proteolytic activity may be accompanied by a down-regulation in antigen uptake via macropinocytosis or Fc-receptors,[38] the loss of PrP^{Sc} degradation by LPS-stimulated XS106 cells may reflect a diminished uptake of the scrapie agent by the cells. Treatment with LPS prior to inoculation with the scrapie agent increases the efficiency of infection.[24] Together these data suggest the effects of LPS treatment on disease pathogenesis might be due to the impaired clearance of the scrapie agent by macrophages or DCs at the site of inoculation. Indeed, depletion of macrophages before peripheral inoculation with the scrapie agent increases the accumulation of infectivity and PrP^{Sc} in the spleen and shortens the incubation period.[39]

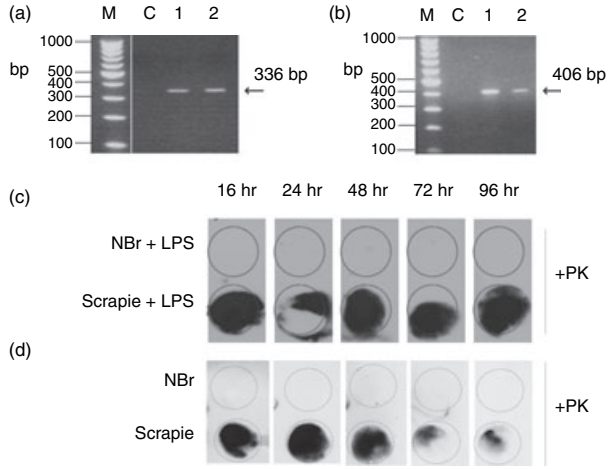

Figure 7. LPS stimulation blocks the degradation of PrP^{Sc} by XS106 cells. (a and b) RT-PCR analysis confirmed that XS106 cells express mRNA specific for TLR-2 (a, lanes 1 and 2, single band at 336 bp) and TLR-4 (b, lanes 1 and 2, single band at 406 bp). Lane M, 100 bp molecular size markers. Lane C, non-reverse transcriptase treated mRNA preparation from XS106 cells as a control. (c) Duplicate cultures of XS106 cells treated with LPS and 1 hr later exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (scrapie) and LPS for 16 hr, washed and then maintained in culture for the times indicated. Cells were then transferred to PVDF membranes and treated in the absence (–) or presence (+) of PK before immunostaining with the PrP-specific mAb 8H4. The PrP^{Sc} associated with LPS-treated scrapie agent exposed XS106 cells was not degraded over the 96-hr observation period (c) when compared to non-LPS treated scrapie agent-exposed XS106 cells (d).

DCs in peripheral tissues, including epidermal LCs, are resting cells that continuously endocytose antigens from their local environment. These cells express very low levels of MHC class II and no costimulatory molecules.[13] Following antigen encounter and appropriate stimulation, LCs become activated and up-regulate the expression of MHC class II, and a variety of costimulatory molecules including CD11c, CD40, CD54 (ICAM-1), CD80 and CD86 on their surfaces transforming the cells from immature to mature LCs.[40–42] This transformation enables the LCs to present endocytosed antigens on their surfaces in association with MHC class II, express costimulatory molecules to interact with T cells and stimulates their migration to draining lymph nodes. In the current study, surface marker expression on XS106 cells did not significantly change following exposure to the scrapie agent, implying that the cells were not specifically activated. These observations are congruent with the demonstration that antigen endocytosis alone does not induce DC activation.[43] These data do not reflect those reported by Bacot *et al.* who have shown that the prion protein peptide $PrP_{106–126}$ induces the up-regulation of HLA-DR, CD40, CD80 and CD83 on the surface of human monocyte-derived DCs.[44] Whether DCs respond differently to recombinant $PrP_{106–126}$ and the scrapie agent is not known. The magnitude of surface marker expression on the XS106 cells used in the current study suggests it is unlikely these cells were terminally mature and could not undergo further maturation following scrapie agent exposure. The effect of scrapie exposure on the surface expression of the C-type lectin CD205 was also analysed as this receptor binds carbohydrate residues and delivers them to lysosomes or late endosomes for degradation.[45] Although these cellular compartments have been proposed as sites for PrP^{Sc} degradation,[46–48] expression of CD205 was similarly unaffected following exposure to the scrapie agent.

Following inoculation of mice with the scrapie agent via the skin, neuroinvasion occurs after the agent accumulates in association with FDCs in the draining lymph node.[5,6] The mechanism through which the scrapie agent is transported to the FDCs within draining lymph node following inoculation via the skin is not known. Upon antigen encounter LCs receive a complex network of signals which stimulate their maturation and migration to draining lymphoid tissue.[13] In mice, inoculation with the scrapie agent does not elicit a specific humoral or cell-mediated immune response.[49,50] Our data above similarly predict that LCs would not become specifically activated following exposure to the scrapie agent in the epidermis. Thus, although LCs have the potential to acquire and degrade the scrapie agent within the epidermis, our data suggest it is unlikely that these cells become activated and are stimulated to transport the agent to the draining lymph node. Indeed, we have recently demonstrated that when LC migration from the epidermis is blocked, scrapie infectivity is still able to reach the draining lymph node following inoculation via the skin suggesting, that LCs are not major TSE agent transporters.[18]

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